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Four new dimeric spiro-azaplilone derivatives cochliodones E-H from the entophytic fungus *Chaetomium* sp. M336

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ABSTRACT

Chaetomium sp. M336 was isolated from *Huperzia serrata* (Thunb. ex Murray) Trev. and subjected to phytochemical investigation based on its special environment. To understand the secondary metabolites of strain *Chaetomium* sp. M336, four new dimeric spiro-azaplilone derivatives cochliodones E-H (**1**–**4**) were isolated from solid fermentation products of the fungus, which were elucidated by extensive spectroscopic analyses, including 1D- and 2D NMR, and HR-ESI–MS experiments. The antibacterial activities of compounds **1**–**4** against bacteria were tested *in vitro*. They exhibited antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* ATCC 6539 and *Enterococcus faecalis* with minimum inhibitory concentration (MIC) values of 0.78–100 μg/mL.

1. Introduction

Endophytic fungi, defined functionally by their occurrence in plant tissue without causing any overt effects (Petrini, 1991; Saikkonen et al., 1998), are present in almost all plants and are important sources of natural products (Stierle et al., 1993; Strobel et al., 2004; Aly et al., 2010). The products of endophytic microbes and their uses in medicine, agriculture and industry have been reviewed (Gutierrez et al., 2012). The increasing number of new compounds discovered in endophytes demonstrates their potential for producing more previously unknown natural products, which are still to be exploited for their potential applications. *Huperzia serrata* (Thunb. ex Murray) Trev., the traditional Chinese medicine Qian Ceng Ta, grows at an altitude of 300–2700 m in damp forests and rock crevices in China (Yang et al., 2003) that produces a lycopodium alkaloid Huperzine A (HupA), which has been marketed in China as a new drug for Alzheimer's disease (AD) treatment and used in the USA as a supplement for preventing further memory degeneration (Liu et al., 1986a,b; Zangara, 2003). In our search for new active compounds from endophytic microorganisms, a series of novel compounds were previously identified. In present work, an endophytic fungus *Chaetomium* sp. M336 was isolated from *Huperzia serrata* Trev. and four new dimeric spiro-azaplilone derivatives cochliodones E-H (Fig. 1) were obtained. This report describes the four new structures and their activities.

2. Results and discussion

The nucleotide sequences for the ITS1-5.8S rDNA-ITS4 region of the endophytic fungus M336 was registered in the GenBank database with the accession number KU743242, and the strain was determined to be *Chaetomium* sp. according to the ITS analysis.

Compound **1** was obtained as yellow oil. The HR-ESI–MS data indicated a molecular formula of $C_{34}H_{38}O_{12}$ based on the $[M+Na]^+$ ion signal at *m/z* 661.2262. The ^{13}C NMR and DEPT spectra (Table 1) revealed eight quaternary carbons, three methines, three methylenes and three methyls. Since the ^{1}H and ^{13}C NMR spectra showed only half the number of resonance signals expected for 38 protons and 34 carbons, the structure must be a symmetrical dimer, which suggested that compound **1** was dimeric spiro-azaplilone derivative (Phonkerd et al., 2008). Comparison with cochliodone A (Phonkerd et al., 2008), O-bridge between C-3/3′ and C-12/12′ was broken to be a line chain and a double bond between C-3/3 and C-4/4 was formed on basis of HMBC

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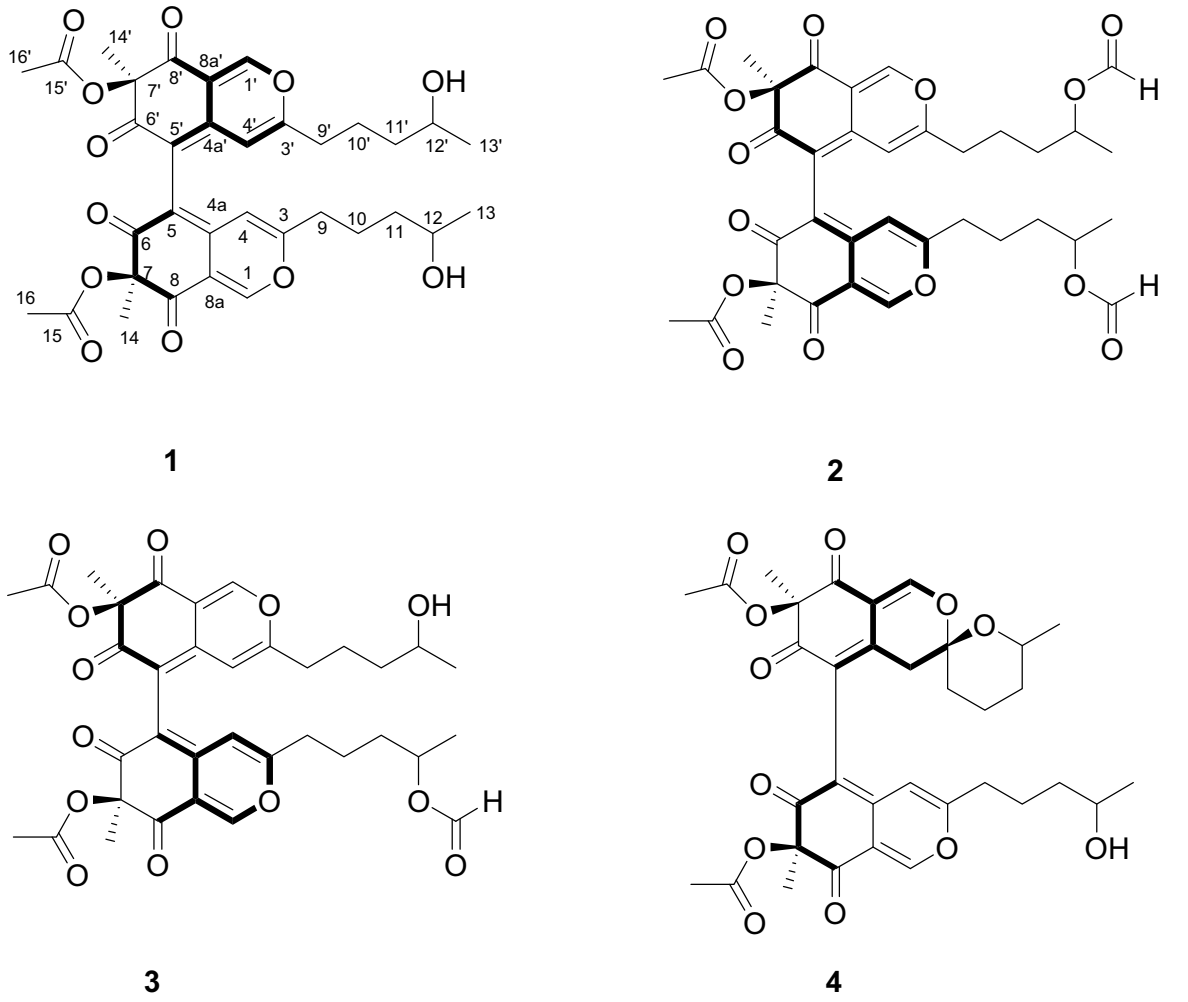

Fig. 1. The structures of compounds **1-4**.

correlations (Fig. 2). In HMBC spectrum, the olefinic proton at δ_H 7.92 (H-1/1′) correlated with the carbons at δ_C 162.7 (C-3/3′), 193.3 (C-8/8′), 142.0 (C-4a/4a') and 115.4 (C-8a/8a'); the olefinic proton at δ_H 5.97 (H-4/4′) with carbons at δ_C 162.7 (C-3/3′), 110.2 (C-5/5′), 193.3 (C-8/8′), 115.4 (C-8a/8a') and 32.9 (C-9/9′); the methylene protons δ_H 2.40 (H-9/9′) with carbons at δ_C 162.7 (C-3/3′), 107.5 (C-4/4′), 22.34 (C-10/10′) and 38.0 (C-11/11′); and the methyl protons at δ_H 1.17 (H-13/13′) with carbons at δ_C 67.4 (C-12/12′) and 38.0 (C-11/11′). Together with COSY (Fig. 2) correlations: fragments were observed to be –C-9/9′–C-10/10′–C-11/11′–C-12/12′–C-13/13′– from a complete interpretation of the monomer correlations. The relative configuration of compound **1** was similar with cochliodone A because the optical rotation of compound **1** ([α]-162 (c 0.17, MeOH) has almost identical with cochliodone A ([α]-226 (*c* 0.14, MeOH)) in the literature (Phonkerd et al., 2008). The configuration of compound **1** is shown in Fig. 1, and named cochliodone E.

Compound **2** was obtained as yellow oil. The HR-ESI–MS data indicated a molecular formula of $C_{36}H_{38}O_{14}$ based on the $[M+Na]^+$ ion signal at *m/z* 717.2159. The MS and NMR spectroscopic data (Table 1) of compound **2** were very similar to those of compound **1** except that one formyloxy group (δ_H 8.01 and δ_C 160.7) in compound **2** instead of one hydroxyl in compound **1** at C-12/12′. The other main HMBC correlations were almost the same as those of compound **1** (Table 1). However, the different physiochemical and specific rotations [**1**, −162 (c 0.17, MeOH); **2**, +15 (c = 0.11, MeOH)] between the two compounds suggested an atropisomeric relationship to each other (Phonkerd et al., 2008). Based on the above data, the configuration of compound **2** is shown in Fig. 1, and named cochliodone F.

Compound **3** was obtained as yellow oil. The HR-ESI–MS data indicated a molecular formula of $C_{35}H_{38}O_{13}$ based on the $[M+Na]^+$ ion signal at *m/z* 689.2214. The MS and NMR spectroscopic data (Table 2) for compound **3** were very similar to those for compound **1** except that one formyloxy group (δ_H 8.01 and δ_C 160.7) in monomeric unit of compound **3** instead of one hydroxyl in compound **1**. So the structure of **3** was not a symmetrical dimmer. The other main HMBC correlations were almost the same as those of compound **1** (Tables 1 and 2) expect that chemical shifts of C-12 were shift to down-field and the formyloxy group was connected with any monomeric unit. However, the different physiochemical and specific rotations [**1**, −162 (c 0.17, MeOH); **3**, +1 (c = 0.12, MeOH)] between the two suggested an atropisomeric relationship to each other. Based on the above data, the configuration of compound **3** is shown in Fig. 1, and named cochliodone G.

Compound **4** was obtained as yellow oil. The HR-ESI–MS data indicated a molecular formula of $C_{34}H_{38}O_{12}$ based on the $[M+Na]^+$ ion signal at *m/z* 661.2261. Comparison with cochliodone A (Phonkerd et al., 2008), an O-bridge between C-3 and C-12 was broken to be a line and a double bond between C-3 and C-4 was formed in monomeric unit on basis of HMBC correlations (Table 2). So the structure of **4** was not a symmetrical dimmer. In addition, the similar physiochemical and specific rotations [cochliodone A, −226 (c 0.14, $CHCl_3$); **4**, −290 (c = 0.14, MeOH)] suggested their

Table 1
NMR data of compounds **1** and **2** (in $CDCl_3$,*J* in Hz).

Position	**1**			**2**		
	^{1}H	^{13}C	HMBC	^{1}H	^{13}C	HMBC
1/1′	7.92 (1H, s)	153.7, d	3/3′, 8/8′, 4a/4a', 8a/8a'	7.92 (1H, s)	153.6, d	3/3′, 8/8′, 4a/4a', 8a/8a'
3/3′	–	162.7, s	–	–	162.1, s	–
4/4′	5.97 (1H, s)	107.5, d	3/3′, 5/5′, 8/8′, 8a/8a', 9/9′	5.95 (1H, s)	107.6, d	3/3′, 5/5′, 8/8′, 8a/8a', 9/9′
4a/4a'	–	142.0, s	–	–	141.8, s	–
5/5′		110.2, s			110.4, s	
6/6′	–	190.4, s	–	–	190.6, s	–
7/7′	–	85.0, s	–	–	84.8, s	–
8/8′	–	193.3, s	–	–	193.4, s	–
8a/8a'	–	115.4, s	–	–	115.5, s	–
9/9′	2.40 (2H, t, 7.6)	32.9, t	3/3′, 4/4′, 10/10′, 11/11′	2.37 (2H, t, 7.0)	32.9, t	3/3′, 4/4′, 10/10′, 11/11′
10/10′	1.76 (1H, m) 1.60 (1H, m)	22.34, t	3/3′, 9/9′, 11/11′, 12/12′	1.61 (2H, m, overlap)	22.08, t	–
11/11′	1.46 (2H, q, 6.2)	38.0, t	9/9′, 12/12′	1.56 (2H, m)	34.9, t	12/12′, 10/10′
12/12′	3.80 (1H, m)	67.4, d	–	5.01 (1H, m)	70.2, d	–
13/13′	1.17 (3H, d, 6.2)	23.3, q	11/11′,12/12′	1.25 (3H, d, 6.5)	19.9, q	11/11′,12/12′
14/14′	1.61 (3H, s)	22.30, q	6/6′, 7/7′, 8/8′	1.61 (3H, m)	20.2, q	6/6′, 7/7′, 8/8′
15/15′	–	170.8, s	–	–	170.4, s	–
16/16′	2.17 (3H, s)	20.3, q	7/7′, 15/15′	2.16 (3H, s)	22.14, q	7/7′, 15/15′
12/12′-CHO	–	–	–	8.01 (1H, s)	160.7, d	12/12′

configurations are the same (Phonkerd et al., 2008). Based on the above data, the configuration of compound **4** is shown in Fig. 1, and named cochliodone H.

Compounds **1**–**4** were assayed for antibacterial activity (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* ATCC 6539 and *Enterococcus faecalis*). All compounds showed antibacterial activities (Table 3). Furthermore, compound **3** could inhibit *E. coli* growth almost the same with cefotaxime.

3. Experimental

3.1. General

UV spectra were measured on a Shimadzu UV-2401PC spectrophotometer, and λ_{max} (log ε) values are reported in nm. NMR experiments were carried out on Bruker Avance 600 NMR spectrometers with tetramethylsilane (TMS) as an internal standard. ESI–MS and HR-ESI–MS were recorded on a Finnigan LCQ-Advantage mass spectrometer and a VG Auto-Spec-3000 mass spectrometer. Optical rotations were measured on a Jasco DIP-370 digital polarimeter. Column chromatography was carried out on silica gel (G, 200–300 mesh and GF_{254}) (Qingdao Marine Chemical Factory, Qingdao, China) and Sephadex LH-20 (Pharmacia). Pre-coated silica gel GF_{254} plates (Qingdao Marine Chemical Factory, Qingdao, China) were used for thin layer chromatography (TLC).

3.2. Fungal material

The plant *Huperzia serrata* (Thunb. ex Murray) Trev., was collected in Xichou County, Yunnan Province, People's Republic of China, in July 2013. A voucher specimen (No. 20130710ZPJ) was deposited at the Herbarium of Kunming Institute of Botany (KUN), Chinese Academy of Sciences. The detail of isolation entophytic fungus was described in previous work (Chen et al., 2015). A strain, designated as M336, appeared after culturing for about two weeks and was isolated from the sterilized branch. The strain was identified to be *Chaetomium* sp. and deposited in Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China. *Chaetomium* sp. M336 (30 L) was cultured on a dish containing PDA (potato 200 g, glucose 20 g, distilled water 1 L, agar 15–20 g, pH is natural) at 25 °C for 17 days.

3.3. Extraction and isolation

The solid fermentation products was cut into small pieces and extracted exhaustively with mixture solution (EtOAc/MeOH/HAc, 80:15:5, v/v/v) by four times to obtain the rude extract. The extract were dissolved in water, and extracted with EtOAc six times, respectively. The extract of ethyl acetate section (40.1 g) residue was subjected to a column of silica gel G (200–300 mesh) using petroleum ether – ethyl acetate (100:4 → 6:4) and $CHCl_3$-MeOH

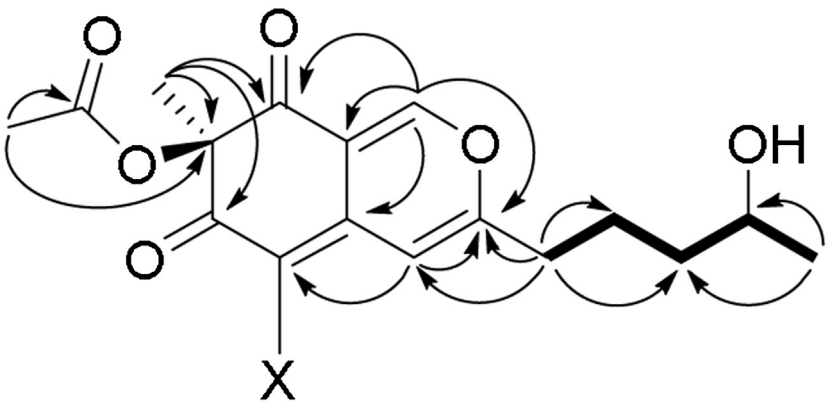

Fig. 2. Key ^{1}H–^{1}H COSY (bold line) and HMBC (arrows) correlations of monomer **1**.

Table 2
NMR data of compounds **3-4** (in $CDCl_3$, *J* in Hz).

Position	**3**			**4**		
	^1H	^{13}C	HMBC	^1H	^{13}C	HMBC
1	7.92 (1H, s)	153.6, d	3, 4a, 8, 5	7.91 (1H, s)	153.6, d	3, 4a, 8, 5
3	–	162.1, s	–	–	162.7, s	–
4	5.96 (1H, s)	107.7, d	3, 5, 8, 8a, 9	5.91 (1H, s)	106.6, d	3, 4a,5, 6, 8a, 9
4a	–	141.88, s	–	–	141.0, s	–
5	–	115.47	–	–	115.2, s	–
6	–	190.4, s	–	–	192.4, s	–
7	–	84.8, s	–	–	84.7, s	–
8	–	193.2, s	–	–	193.0, s	–
8a	–	110.5, s	–	–	110.9, s	–
9	2.39 (2H, q, 7.3)	33.0, t	3, 4, 10, 11	1.88 (1H, m); 1.43 (1H, m)	31.7, t	3
10	1.62 (2H, m)	22.1, t	–	1.43 (2H, m)	22.15, t	9, 12
11	1.62 (2H, d, 3.8)	34.9, t	–	1.63 (1H, m); 1.24 (1H, d, 1.5)	31.3, t	12
12	5.03 (1H, m)	70.2, d	–	3.90 (1H, m)	68.7, d	–
13	1.25 (3H, overlap)	19.9, q	11, 12	1.10 (3H, d, 6.2)	21.6, q	11, 12
14	1.61 (3H, s)	22.2, q	6, 7, 8	1.60 (1H, s)	22.18, q	6, 7, 8
15	–	170.4, s	–	–	170.1, s	–
16	2.16 (3H, s)	20.2, q	7, 15	2.16 (3H, s)	20.24, q	7, 15
12-CHO	8.02 (1H, s)	160.7, d	12	–	–	–
1′	7.93 (1H, s)	153.6, d	3′, 4a', 5′, 8′	7.80 (1H, s)	158.1, d	3′, 4a', 5′, 8′
3′	–	162.7, s	–	–	103.3, s	–
4′	5.98 (1H, s)	107.5, d	3′, 5′, 8′, 8a', 9′	2.73 (1H, d, 16.7); 2.50 (1H, d, 16.7)	37.4, t	3′, 8′, 8a',9′
4a'	–	141.89, s	–	–	145.8, s	–
5′	–	115.49, s	–	–	122.2, s	–
6′	–	190.5, s	–	–	190.1, s	–
7′	–	84.9, s	–	–	84.8, s	–
8′	–	193.4, s	–	–	192.2, s	–
8a'	–	110.3, s	–	–	110.6, s	–
9′	2.39 (2H, q, 7.3)	32.9, t	–	2.39 (2H, t, 8.4)	32.9, t	4′
10′	1.62 (2H, m)	22.1, t	–	1.69 (2H, m)	18.2, t	9′, 12′
11′	1.46 (2H, q, 6.7)	38.1, t	9′, 12′, 13′	1.43 (2H, m)	37.9, t	9′, 12′
12′	3.79 (1H, m)	67.5, d	–	3.78 (1H, m)	67.3, d	–
13′	1.18 (3H, d, 6.2)	23.4, q	11′, 12′	1.16 (3H, d, 6.2)	23.4, q	12′
14′	1.61 (3H, s)	22.3, q	6′, 7′, 8′	1.61 (1H, s)	22.18, q	6′, 7′, 8′
15′	–	170.8, s	–	–	170.6, s	–
16′	2.18 (3H, s)	20.3, q	7′, 15′	2.16 (3H, s)	20,19, q	7′, 15′

(100:6 → 0:100) gradient solvent system to produce 11 fractions (Fr.1–Fr.11).

The fraction Fr.6 (2.5 g) was subjected to a column of silica gel G (200–300 mesh) using $CHCl_3$-MeOH (100:0 → 6:4) gradient solvent system to give 7 fractions of Fr.6.1 to Fr.6.7. The Fr.6.2 (385.2 mg) was subjected on Sephadex LH-20$CHCl_3$-MeOH (1:1) to produce 3 fractions (Fr.6.2.1–Fr.6.2.3). The Fr.6.2.1 (199.5 mg) was subjected to a column of silica gel (GF_{254}) using $CHCl_3$-MeOH 100:4 → 6:4) gradient solvent system to produce 4 fractions (Fr.6.2.1.1–Fr.6.2.1.4). The Fr.6.2.1.2 (151.3 mg) was purified using Sephadex LH-20 (C_3H_6O) to produce 3 fractions (Fr.6.2.1.2.1–Fr.6.2.1.2.3). The Fr.6.2.1.2.1 (69.4 mg) was dissolved in acetone and then repeated subjected to preparational TLC separating plates (GF_{254}), particular developing agent [$CHCl_3$/Acetone 4:1 (100 mL) and 0.3 mL of CH_3COOH per plate]. The preparational TLCs were run twice for best separation, and densitometric analyses of the chromatogram were carried out with a ternary wavelength TLC scanner ZF-I at 254 nm. The product (Fr.6.2.1.2.1.1) was removed from the plates by elution of the scratched zones with the particular developing agent. The Fr.6.2.1.2.1.1 (45.8 mg) was purified using semipreparative HPLC (NP7000 Semi-preparation Gradient HPLC System, China) to produce **4** (20.3 mg). The Fr.6.2.1.2.3 (49.2 mg) was isolated using semipreparative HPLC (NP7000 Semi-preparation Gradient HPLC System, China) to produce **3** (4.4 mg) and **1** (9.5 mg). The fraction Fr.9 (6.1 g) was subjected to a column of silica gel G (200–300 mesh) using $CHCl_3$-MeOH (100:6 → 0:100) gradient solvent system to give 13 fractions of Fr.9.1 to Fr.9.13. The Fr.9.6 (1.8 g) was isolated using Sephadex LH-20$CHCl_3$-MeOH (1:1) to produce 4 fractions (Fr.9.6.1–Fr.9.6.4). The Fr.9.6.2 (633.0 mg) was subjected to a column of silica gel G (200–300 mesh) using $CHCl_3$—C_3H_60 (10:1 → 0:100) gradient solvent system and subjected to a column of silica gel (GF_{254}) using $CHCl_3$-MeOH (100:3 → 0:100) to produce **2** (55.7 mg).

3.3.1. Cochliodone E (**1**)

Yellow oil; [α] = −162 (c = 0.17, MeOH); UV (MeOH) λ*max* (log ε): 203 (4.93), 221 (4.51), 332 (4.51); NMR data see Table 1; ESI–MS: 661 $[M+Na]^+$; HR-ESI–MS: 661.2262 ($[M+Na]^+$, calc. 661.2255).

3.3.2. Cochliodone F (**2**)

Yellow oil; [α] = +15 (c = 0.11, MeOH); UV (MeOH) λ_{max} (log ε): 221 (4.63), 335 (4.60), 467 (3.46); NMR data see Table 1; ESI–MS: 717 $[M+Na]^+$; HR-ESI–MS: 717.2159 ($[M+Na]^+$, calc. 717.2154).

3.3.3. Cochliodone G (**3**)

Yellow oil; [α] = +1 (c = 0.12, MeOH); UV (MeOH) λ_{max} (log ε): 220 (4.53), 333 (4.43), 468 (3.38); NMR data see Table 2; ESI–MS: 667 $[M+H]^+$; HR-ESI–MS: 689.2214 ($[M+Na]^+$, calc. 689.2205).

3.3.4. Cochliodone H (**4**)

Yellow oil; [α] = −290 (c = 0.14, MeOH); UV (MeOH) λ_{max} (log ε): 204 (4.46), 328 (4.49), 468 (3.48); NMR data see Table 2; ESI–MS: 639 $[M+H]^+$; HR-ESI–MS: 661.2261 ($[M+Na]^+$, calc. 661.2255).

3.4. Assay activities

The antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* ATCC 6539 and *Enterococcus faecalis* was assayed. The MICs for antibacterial activity were determined by a method modified from the standardized microdilution method (Tereshuck et al., 1997). Four bacteria were incubated in Nutrient Agar at 25 °C for 18 h overnight. A few

Table 3
The antibacterial activities of **1–4** (μg/mL).

Compounds	*E. coli*	*Staphylococcus aureus*	*Salmonella typhimurium* ATCC 6539	*Enterococcus faecalis*
1	50	NA[a]	100	NA
2	50	NA	50	50
3	0.78	NA	50	100
4	50	NA	100	NA
Cefotaxime	0.19	0.19	1.56	1.56

[a] NA means it did not show antibacterial activities at 100 μg/mL.

colonies of bacteria were collected aseptically with a sterile loop and introduced into 10 mL of sterile 0.9% saline solution. The concentration of the suspension was then standardized by adjusting the optical density to 0.10 at 630 nm, corresponding to bacterial cell suspension of 10^8 colony-forming units/mL (CFU/mL). This cell suspension was diluted 100 times to obtain 10^6 CFU/mL before use. All of the tested compounds were dissolved in DMSO to make stock solutions of 4 μg/μL. Double dilution was used. Different doses of the test compounds (ranging from 100 to 0.78 μg/mL) and broth were added to each well (Deepwell Plates 96, Eppendorf) containing bacteria suspension (100 μL) and maintained at a final volume of 200 μL (the final concentration of 5% (v/v) DMSO or less). DMSO was used as a negative control, and the bacterial solution without the compound and DMSO was used as a blank control. Cefotaxime was used as a positive control. Deepwell plates containing the various components were incubated at 37 °C for 24 h. After incubation, the wells were examined for growth of microorganisms by measuring optical density (OD) values of the wells. The MIC is the lowest concentration of the test compound at which no visible growth occurs. All of the experiments were performed in triplicate.

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